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Authors: Bates, William R., and Nishida, Hiroki

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Developmental Roles of Nuclear Complex Factors Released during Oocyte Maturation in the Ascidians *Halocynthia roretzi* and *Boltenia villosa*

William R. Bates[1,2]* and Hiroki Nishida[2]

[1]*Bamfield Marine Station, Bamfield, British Columbia, Canada VOR 1BO and the Department of Biology, University of Victoria, Victoria, British Columbia, Canada V8W 2Y2*
[2]*Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226, Japan*

ABSTRACT—The developmental roles of factors associated with the nuclear complex of *Halocynthia roretzi* and *Boltenia villosa* oocytes were investigated by cutting mature oocytes into animal and vegetal merogons before and during GVBD. Animal and vegetal merogons were cultured in sea water until the GV cytoplasm had dispersed within the cytoplasm of control oocytes and then they were cross-fertilized and scored for their ablility to undergo normal development. *Halocynthia* oocyte fragments produced from the animal region of oocytes containing intact GVs exhibited a low frequency of polyspermy, a high frequency of fertilization and cleavage, and a high frequency of expressing an epidermal antigen, Epi-2. In contrast, merogons produced from the vegetal region of *Halocynthia* oocytes in which GVs were intact exhibited a high frequency of polyspermy, did not undergo cell division, and expressed a high frequency of Epi-2 expression. When vegetal fragments were produced after the dispersal of approximately 50-70% of the GV nucleoplasm, these merogons exhibited a low frequency of polyspermy, high frequencies of cell division (including the formation of epidermal layer), and in most cases expressed Epi-2. Vegetal *Boltenia* fragments produced during GVBD in some cases developed into larvae. These results suggest that the ascidian GV nucleoplasm may contain factors required for fertilization and cell division and that epidermal determinants reside in the oocyte cytoplasm.

INTRODUCTION

Maternal RNA molecules and proteins are known to be sequestered in the nuclear complex of animal oocytes that are subsequently released into the cytoplasm during maturation. In amphibian oocytes, factors derived from the germinal vesicle have been shown to persist in the cytoplasm of embryonic cells and affect their development (Huff, 1962; Briggs and Cassens, 1966; Malacinski, 1974). One of these factors has recently been identified in *Xenopus* oocytes, a histone-binding protein, N1, that is initially stored in the nuclear complex of the oocyte and is then dispersed throughout the cytoplasm after the dissolution of the GV membrane. Surprisingly, N1 was shown to re-enter nuclei of particular cell-lineages during cleavage suggesting that N1 may function in determining the fates of the recipient cells (Dreyer *et al*., 1981, 1982; Dreyer, 1987). Results obtained by Kleinschmidt *et al*. (1985) suggest that N1 mediates the assembly of nucleosomes, thereby altering the cell's state of determination.

In ascidians, only a few studies have examined the potential roles of factors associated with the GV. The nucleosome modification model suggested by Dreyer *et al*. (1981) and Kleinschmidt *et al*. (1985) was examined in ascidians by Fujiwara and Satoh, who exploited the autonomous and fixed cell lineage mode of development that is typical of the ascidian embryo (Fujiwara and Satoh, 1990; Fujiwara, 1993; Fujiwara *et al*., 1993). In the ascidian *Halocynthia roretzi*, a 83-kDa nuclear protein, termed Hgv2, that is closely related to the amphibian N1 histone-binding protein, was found to be localized in the oocyte GV. However, when it was subsequently discovered that Hgv2 was present in all embryonic nuclei throughout development, it appeared that Hgv2 is not likely involved in the determination of ascidian cell fates.

Another factor has been suggested to be associated with the germinal vesicle cytoplasm of the ascidian oocyte (Conklin, 1905). When Conklin traced the basophilic staining pattern of the GV nuclear complex after GVBD in fertilized eggs, he observed that the GV cytoplasm was partitioned selectively into the epidermal cell lineage of the embryo. For this reason Conklin referred to the contents of the ascidian GV as "ectoplasm". The chemical basis of the basophilic staining of GV cytoplasm first described by Conklin was discovered by more recent experiments in which the distributions of maternal

* Corresponding author: Tel. +1-250-728-3267;
FAX. +1-250-728-3452.

poly(A)$^+$ RNA were examined in ascidian oocytes using in situ hybridization (Jeffery and Capco, 1978). These experiments indicate that approximately 45% of the total poly(A)$^+$ RNA is contained within the GV of postvitellogenic stage III oocytes (Jeffery and Capco, 1978). These results together with those obtained from experimental manipulations of ascidian eggs (Reverberi and Minganti, 1946; Nishikata *et al*., 1987; Nishida, 1992, 1994; Yamada and Nishida, 1996) suggest the possibility that epidermal determinants, perhaps oogenetic RNA, are responsible for the autonomous development of epidermal progenitor cells.

Several studies have examined the development of epidermal cells in ascidian embryos. The approximately eight hundred epidermal cells that surround an ascidian larva (Monroy, 1979) are derived from the animal hemisphere cytoplasm of the fertilized egg which is partitioned into epidermal progenitor cells prior to first cleavage (Conklin, 1905; Nishida and Satoh,1983; Nishida, 1987).

At the four-cell stage, each blastomere produces epidermal cells, however, after the equatorial third cell division, only the animal blastomeres of an eight-celled embryo, designated the a4.2 and b4.2 blastomeres, produce epidermal cells. The recent cloning of several epidermal-specific genes in *Halocynthia* (Ueki *et al*., 1991) will hopefully facilitate molecular investigations how this partitioned animal cytoplasm determines epidermal cell fates.

It is known that ascidian epidermal cell fates are autonomously determined. The autonomous differentiation of epidermal lineage cells in *H. roretzi* has been investigated using an epidermis-specific antibody, termed Epi-2 (Mita-Miyazawa *et al*., 1987). Cleavage-arrested zygotes and isolated a4.2 and b4.2 epidermis progenitor blastomeres have been shown to express the Epi-2 antigen (Mita-Miyazawa *et al*., 1987; Nishikata *et al*., 1987; Nishida, 1992). These results support the idea that epidermal cell differentiation requires cytoplasmic factors that reside in the unfertilized ascidian egg. However, as unfertilized eggs were used in these previous experiments, the aim of the present study was to explore the possibility that epidermal factors and other factors may be associated with the nuclear complex of the ascidian GV.

The results of the present study suggest that the ascidian GV contains factors required for fertilization and cell division and that factors required for the differentiation of an epidermal cell marker, Epi-2, reside in the cytoplasm of mature ascidian oocytes.

MATERIALS AND METHODS

Adult ascidians and collection of gametes

Halocynthia roretzi adults were collected by fishermen in the vicinity of Asamushi Marine Biological Station, Mutsu Bay, Aomori, Japan during the breeding season. Pieces of gonad were removed from adults by dissection and stored "dry" (that is, they were not immersed in sea water) at 10°C. Sperm from two or more adults was collected for the inseminations. Immediately before performing a microsurgery experiment, *Halocynthia* oocytes at various stages of maturity were dissected from ovaries and washed with large volumes of sea water. Sperm was added to culture dish wells for inseminations.

Boltenia villosa adults, a species common to the southern coast of Vancouver Island, British Columbia, Canada were purchased from Westwind Sealab Supplies, Victoria, British Columbia, Canada. Adults were maintained in natural flowing sea water at 10–11°C. Oocytes and sperm were obtained by the dissection of gonads. Sperm from two or more adults was collected for the fertilization of mature oocytes. Oocytes at various stages of maturation were collected immediately before the microsurgical operations and washed with large volumes of sea water.

Oocyte microsurgery

A detailed description of oogenesis in *H. roretzi*, reported by Fuke and Numakunai (1996), was used for the selection of mature oocytes in this species. The selection of mature *Boltenia* oocytes for microsurgical operations from mixed populations of oocytes at different stages of oogenesis was based on descriptions of oogenesis in *Styela* (Jeffery and Capco,1978) and in *Ascidia nigra* (Cowden, 1961). Mature stage III *Boltenia* oocytes have the largest diameters, fully enlarged GVs, and test cells within the perivitelline space.The morphological features of type 3 ovarian "eggs" (OVE3) in *Halocynthia* corresponded to mature stage III oocytes in *Boltenia*, except that the perivitelline space of mature *Halocynthia* oocytes was reduced, as compared to stage III *Boltenia* oocytes. In the present report, the term "oocyte" is used prior to GVBD and "egg" is used after GVBD.

GVs were removed from OVE3 oocytes of *H. roretzi* using a modification of the "extrude and cut" method previously described by Bates and Jeffery (1987b). As the plasma membranes of *Halocynthia* oocytes were closely adhered to the chorion making extrusion very difficult, oocytes were treated with 0.05% actinase E dissolved in sea water for 30 sec followed by extensive washing with sea water. This treatment resulted in the formation of a narrow perivitelline space that facilitated the extrusion of either the animal or vegetal regions of an oocyte. In both species, the follicular envelope (FE) is required for fertilization, therefore, GV-minus (vegetal) or GV-plus (animal) fragments were produced within the FE for subsequent fertilization.

The microsurgical method that was used to make oocyte fragments is described as follows. An oocyte was immobilized on the bottom of a dish using the tip of an oil-stone sharpened insect pin. To produce a vegetal (GV-minus) fragment within the FE, a tear corresponding to approximately the diameter of a GV was made in the FE above the animal pole region of an oocyte. When pressure was applied to the vegetal pole region using the tip of an insect pin, the animal hemisphere containing the GV was extruded through the tear in the FE. In the final step, the cytoplasmic bridge that connected the extruded and non-extruded oocyte regions was cut using the tip of an insect pin. To check that GV membranes were intact after extrusion in the production of GV-minus vegetal fragments, GVs were dissected from the cytoplasm of isolated animal fragments. Isolated GVs were subsequently cultured in wells containing sea water to determine if they could undergo GVBD in the absence of cytoplasmic factors contained in the oocyte.

As it is known that the immersion of ascidian oocytes in sea water triggers the resumption of meiotic maturation (Fuke and Numakunai, 1996), oocyte fragments were produced before and during GVBD and cultured in sea water until 90-100% of the unoperated oocytes completed GVBD, thus allowing sufficient time for meiosis to resume before being inseminated with non-self sperm.

To examine the role of maternal factors sequestered in the GV and subsequently released during GVDB, oocyte fragments were produced before and during GVDB. GV-plus animal hemisphere fragments and GV-minus vegetal hemisphere fragments were produced 5 to 20 min after immersion of oocytes in sea water, corresponding to the time when GV membranes were intact, as confirmed by dissecting oocytes at various times during the course of their maturation in sea water. Vegetal oocyte fragments were also produced during GVBD corresponding to the time when approximately 50-70% of the GV nucleoplasm had dispersed within the ooplasm, estimated by mea-

suring the diameter of the dispersing GV plasm within the animal pole region.

Specimens were transferred to plastic tissue culture dishes and cultured until the unoperated controls developed into hatched larvae, corresponding to approximately 32 to 36 hr of development at 10-11°C.

Immunocytochemistry

Specimens were fixed for wholemount immunocytochemistry, as previously described by Mita-Miyazawa *et al.* (1987). Animal and vegetal fragments produced from *H. roretzi* oocytes were stained with the epidermal cell monoclonal antibody, Epi-2 (clone 4C5B7, Nishikata *et al.*, 1987), after control eggs developed into hatched larvae. Epi-2 labelled cells were observed using IgG-FITC diluted 1:300 in PBS followed by epifluorescence microscopy.

RESULTS

The time course of GVBD in *Halocynthia* oocytes was examined for oocytes that were surgically dissected from ovaries and immersed in sea water. Oocytes within the ovary were all arrested in meiosis, as indicated by the presence of a GV. However, when oocytes were immersed in sea water for approximately 1 hr, meiotic maturation resumed, as indicated by the breakdown of the GV membrane (Table 1). This time course for GVBD was used for the production of oocyte fragments.

Figure 1A shows a mature *Halocynthia* oocyte in which the animal region was extruded through a tear made in follicular envelope. An intact GV is evident within the extruded region of the oocyte. By cutting the cytoplasmic bridge connecting the extruded and non-extruded regions, a vegetal fragment within the follicular envelope is produced that lacks a GV. To check that the GV membrane was intact after microsurgery, GVs were dissected from the extruded animal regions and cultured in sea water. Figure 1B shows an intact GV dissected from an extruded animal region and cultured for two hours in sea water. Isolated GVs cultured in sea water failed to undergo GVBD (Table 1). GVs dissected from extruded animal fragments during the production of vegetal fragments were cultured in sea water and none of the 25 isolated GVs that were cultured underwent GVBD.

The process of GVBD in living *H. roretzi* oocytes is shown in Fig. 2. Based on the timing of GVBD in oocytes dissected from the ovary and cultured in sea water (Table 1), animal and vegetal fragments were produced prior to GVBD, as shown in Fig. 2A (operation #1) or from oocytes that had initiated GVBD, as shown in Fig. 2B (operation #2). Fragments produced prior to GVBD were generated within 5-10 min after being immersed in sea water. In operation #2, fragments were produced after approximately 50-70% of the GV nucleoplasm had dispersed, corresponding to approximately 45 min after their immersion in sea water (Table 1).

The results describing the insemination and subsequent development of *Halocynthia* oocyte fragments are presented in Tables 2 and 3. When oocytes were cut into animal and vegetal merogons prior to GVBD, significant differences were observed in the relative frequencies of fertilization, polyspermy and cell division. In animal fragments, forty percent of the fragments were unfertilized, 28% were polyspermic (formed multiple nuclei and sometimes exhibited pseudocleavage) and 32% underwent cleavage. Sixteen percent of the cleaved merogons developed into permanent blastulae with an outer epidermal layer and sixteen percent of cleaved merogons developed into larvae.

In contrast, all 17 of the vegetal merogons tested were polyspermic (100%) and none of these fragments underwent cell division. When animal and vegetal fragments were examined for the differentiation of an epidermal cell antigen, Epi-2, both of these types of merogons could develop this epidermal cell marker. Figure 3A shows a partial embryo derived from the animal region of an oocyte stained with Epi-2 in which epidermal cells are stained. In Fig. 3B, an uncleaved, polyspermic fragment produced from the vegetal region of an oocyte prior to GVBD is shown stained positive for Epi-2.

As the results of operation #1 (merogons made prior to GVBD) could suggest that components associated with the GV nucleoplasm promote normal fertilization and cell division, vegetal fragments were produced during GVBD after approximately 50-70% of the GV nucleoplasm had been dispersed (operation #2). The results of these operations are presented in Table 2. Five percent of these fragments were unfertilized, 14% were polyspermic, and 76% of these vegetal fragments underwent cell division. The timing of the first three cell divi-

Table 1. GVBD in *Halocynthia* oocytes and isolated GVs

GVBD within the ovary: 0/420 (0%)
GVBD in intact oocytes immersed in SW for 1 hr: 253/266 (95%)
Duration required for approx. 50-70% of GV cytoplasm to be dispersed: a. SW immersion only: 45 - 50 min after immersion (N= 116) b. Trypsin-treatment in SW for 30 sec: 30 min after immersion (N=135) Time for ~ 50% to100% dispersion of GV cytoplasm: 4 to 6 min (N=40)
GVBD in dechorionated oocytes immersed in SW for 1 hr: 14/14 (100%)
GVBD in isolated GVs immersed in SW for 2 hr: 0/25 (0%)

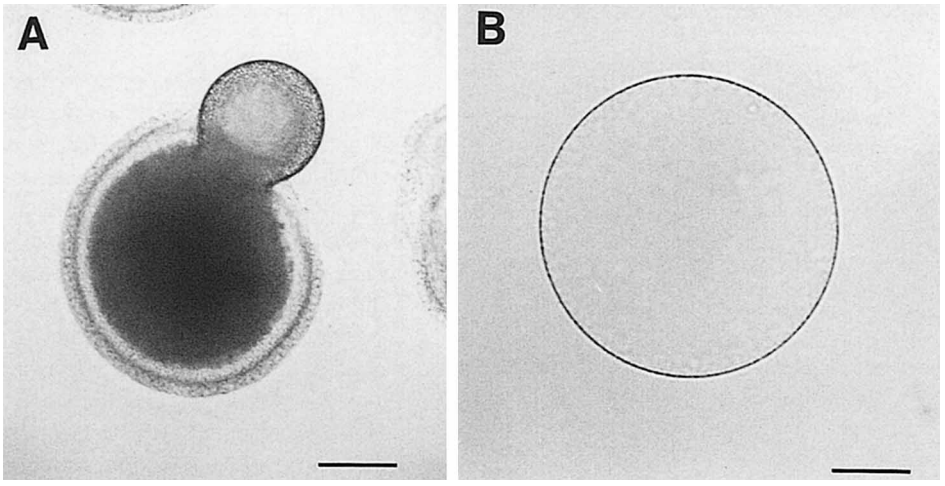


Fig. 1. Light micrographs of an extruded *Halocynthia roretzi* oocyte (**A**) and an isolated *H. roretzi* germinal vesicle cultured for 2 hr in sea water (**B**). In (**A**), the animal region of an oocyte containing an intact germinal vesicle is shown extruded through a tear made in the follicular envelope. A GV-minus fragment within the follicular envelope is produced by cutting the cytoplasmic bridge that connects the extruded and non-extruded oocyte regions. Scale bar in (**A**) equals 100 μm; (**B**) equals 25 μm.

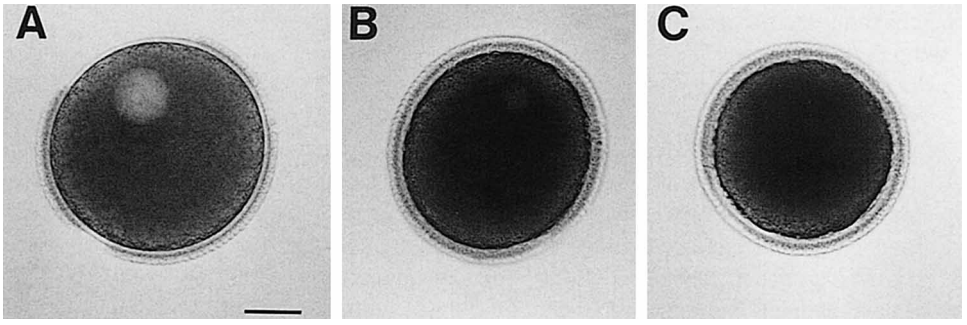


Fig. 2. Light micrographs showing GVBD in *H. roretzi* oocytes. (**A**) A mature oocyte is shown containing a prominent germinal vesicle eccentrically positioned in the animal region of the oocyte. (**B**) This oocyte is undergoing GVBD in which approximately 50-70% of the germinal vesicle cytoplasm has dispersed. (**C**) This oocyte has completed GVBD. Scale bar in (**A**) equals 100 μm; same magnification in each frame.

sions of vegetal fragments was normal (Table 3). In 29% of the cases tested, these fragments developed into permanent blastulae with an epidermal layer. In one case, an abnormal larva developed. When these merogons were stained for Epi-2, after the controls developed into hatched tadpoles, 81% of them stained positive for the Epi-2 antigen.

The results of experiments using *Boltenia* oocytes are shown in Figs. 4 and 5 and in Table 4. Figure 4 shows a pair of oocyte fragments produced from the same *Boltenia* oocyte after approximately 50-70% of the GV nucleoplasm had dispersed, corresponding to the time of operation #2 using *Halocynthia* oocytes. Operation #1 was not done with *Boltenia* oocytes and the expression of epidermal markers was not examined in this species because antibodies were not available. The larger fragment shown in Fig. 4 resides within the follicular envelope and is therefore able to be fertilized, whereas the smaller animal hemisphere fragment corresponds to the extruded region containing approximately half of the GV nucleoplasm.

Table 4 presents the results that were obtained using animal and vegetal *Boltenia* fragments. Twenty-four percent of the vegetal merogons (50 examined in total) were unfertilized, 22% were polyspermic, 54% initiated normal cleavage and 34% developed into larvae (Fig. 5). Sixty-eight percent of the animal hemisphere merogons were unfertilized, 10% were polyspermic, and 22% developed into larvae. The present results are summarized in Table 5 and Fig. 6.

DISCUSSION

The present results provide direct experimental support for the idea that the ascidian GV may contain diffusible factors required for normal fertilization and cell division and that larval epidermal determinants exist in the oocyte cytoplasm. These conclusions are supported by three observations: first, GV-minus vegetal merogons produced prior to GVBD exibited a high rate of polyspermy and did not undergo normal cell division (in some cases, multiple pseudocleavage appeared to have occurred), whereas GV-plus animal merogons exhibited a low rate of polyspermy and a high rate of normal cleav-

Table 2. Development of fragments produced from *Halocynthia roretzi* oocytes

	Early operations (before GVBD)		Late operations (during GVBD)
	Animal merogons	Vegetal merogons	Vegetal merogons[1]
Number examined:	25	17	21
Number unfertilized:	10 (40%)	0 (0%)	1 (5%)
Number polyspermic:	7 (28%)	17 (100%)	3 (14%)
Number cleaved:	8 (32%)	0 (0%)	16 (76%)
Number perm. blast. with epi:	4 (16%)	0 (0%)	6 (29%)
Number larvae:	4 (16%)	0 (0%)	1 (5%)
Number Epi-2 positive:	15 (60%)	14 (82%)	17 (81%)

[1] One merogon cytolyzed.

Table 3. Timing of initial cell divisions in partial *Halocynthia roretzi* embryos produced from the vegetal region of oocytes during the early phase (operation #2) of GVBD

	1st	2nd	3rd
GV-deficient embryos: (n=10)	~ 3 hr	~ 1 hr	~ 1 hr
Control embryos: (n=30)	~ 3 hr	~ 1 hr	~ 1 hr

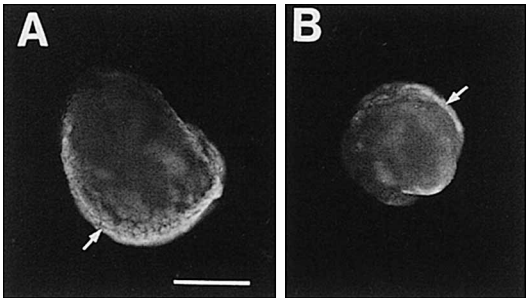


Fig. 3. Immunofluorescence micrographs of animal and vegetal *H. roretzi* oocyte fragments stained with Epi-2 antibody after controls developed into larvae. (**A**) A partial embryo derived from the animal region containing GV cytoplasm showing Epi-2 positive epidermal cells (arrow). (**B**) A single-celled, polyspermic GV-minus fragment produced from the vegetal region of an oocyte in which the GV was intact showing Epi-2 staining (see arrows). Scale bar in (**A**) equals 100 μm; same magnification in each frame.

age; second, GV-minus merogons produced during GVBD exhibited a lower rate of polyspermy and a higher rate of cell division, as compared to GV-minus fragments produced prior to GVBD; and three, both GV-minus vegetal fragments and GV-plus animal fragments expressed the Epi-2 antigen. These results raise the possibility that diffusible GV nucleoplasm factors may be involved. However, polyspermy may somehow evoke merogonal Epi-2 expressions. This intriguing possibility is now being explored. Furthermore, as polyspermic cytoplasm would likely affect normal cell division processes in vegetal fragments produced before GVBD, it will be important to test non-polyspermic vegetal merogons, when the conditions required for their production are discovered.

While keeping in mind that polyspermic effects may be real, the present results suggest the possibility that GVBD is required for the generation of normal fertilization potentials and calcium waves that have been studied in detail in ascidians (Dale *et al*., 1983; Goudeau *et al*., 1992; Speksnijder *et al*., 1989, 1990; Talevi and Dale, 1986). In starfish (Yamada and Hirai, 1984) and mice (Balakier and Tarkowski, 1980), it has been suggested that a nucleoplasmic component released during GVBD may be required for male pronuclear development. Their results provide a plausible explanation why fertilized *Halocynthia* oocyte fragments produced from the vegetal region of oocytes prior to GVBD were polyspermic and failed to undergo normal cell division. The present results also provide evidence for a previous idea that GVBD prevents polyspermy in *Halocynthia* (Fuke and Numakunai, 1996). A cdc2-like kinase has recently been implicated in maturation of ascidian oocytes (Russo *et al*., 1996). In the present study, the inability of a GV to undergo GVBD when it is removed from the cytoplasm of an oocyte and subsequently cultured in sea water is consistent with a putative role for cdc2 kinase in ascidian oocyte maturation. While the present results directly support the results reported by Fuke and Numakunai (1996) that GVBD is required for normal fertilization, the present results support, only indirectly, that GVBD is required for normal fertilization potentials and calcium waves.

The present results re-address the possibility that Hgv2 proteins stored in the GV of *Halocynthia* oocytes (Fujiwara and Satoh, 1990) may be involved in cell division. Most types of mRNA in a sea urchin oocyte reside in the cytoplasm, with the noteable exception of histone mRNA that is squestered in the GV until after GVBD when it is then mobilized into polysomes and translated (Showman *et al*., 1982; DeLeon *et al*., 1983). In striking contrast, histone RNA has been shown to be distributed uniformly between the GV plasm, endoplasm and myoplasm regions in ascidian oocytes, fertilized eggs and two-celled embryos (Jeffery *et al*., 1983). Furthermore, histone mRNA, actin mRNA and a yellow crescent RNA have been shown to be associated with specialized cytoskeletal domains present in the egg cytoplasm (Jeffery, 1984; Swalla and Jeffery, 1995). What is most intruging, is that the nuclear matrix of an ascidian GV contains about half of the total poly (A) RNA content of an oocyte, as measured using ^{3}H-poly (U) probes (Jeffery and Capco, 1978; Jeffery, 1984). When this pool of RNA is removed from an oocyte (as in operation #1 in the present study), the resulting merogons are polyspermic and fail to undergo normal cell division.

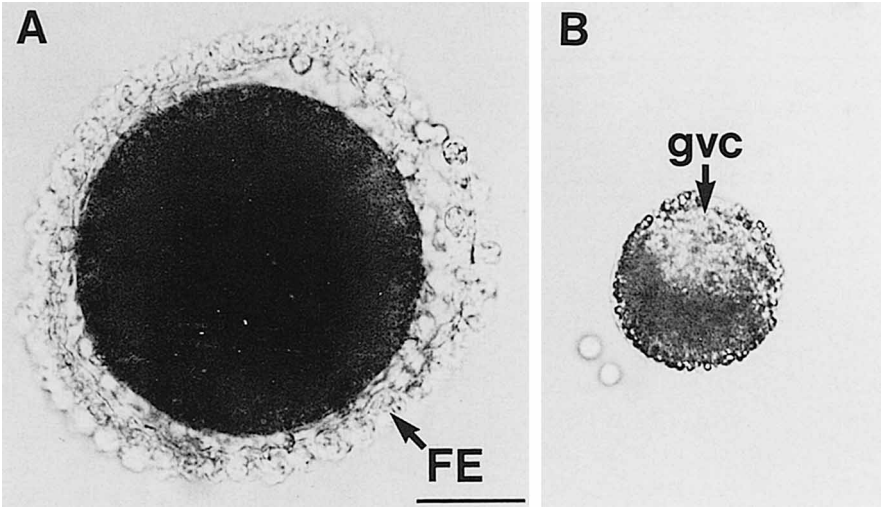


Fig. 4. Light micrographs of two fragments produced from the same mature *Boltenia villosa* oocyte. (**A**) A vegetal oocyte fragment within the follicular envelope produced during the early phase of GVBD. (**B**) An animal oocyte fragment produced from the same oocyte as shown in (**A**) in which the GV membrane has dissolved and part of the peripheral nucleoplasm has dispersed. FE, follicular envelope; gvc, GV. Scale bar in (**A**) equals 50 μm; same magnification in each frame.

Table 4. Development of animal and vegetal fragments produced from *Boltenia villosa* oocytes during the early phase of GVBD (operation #2)

	Animal fragments	Vegetal fragments	Unoperated controls[1]
Number examined:	31	50	33
Number unfertilized:	21 (68%)	12 (24%)	7 (21%)
Number polyspermic:	3 (10%)	11 (22%)	0 (0%)
Number cleaved:	7 (22%)	27 (54%)	23 (70%)
Number perm. blastula with epidermis:	0 (0%)	0 (0%)	0 (0%)
Number of larvae:	7 (22%)	17 (34%)	23 (70%)

[1] Three merogons cytolyzed.

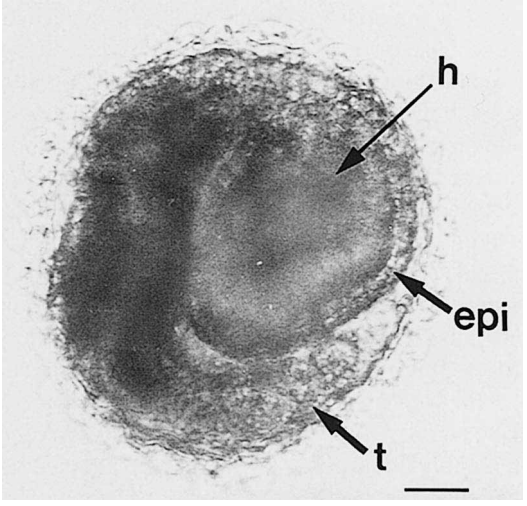
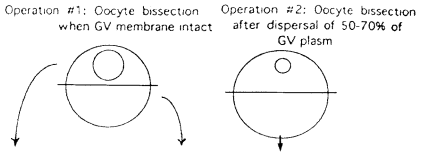


Fig. 5. A light micrograph of a *B. villosa* larva that developed from a vegetal oocyte fragment produced from an oocyte undergoing the early phase of GVBD in which approximately 50% of the germinal vesicle cytoplasm has dispersed. The dispersal of peripheral nucleoplasm GV factors into the vegetal region during the early phase of GVBD resulted in vegetal oocyte fragments that could undergo normal fertilization and cell division and develop into larvae. In this larva, head and tail regions are evident and epidermal cells surround the entire larva. epi, epidermis; t, tail; h, head. Scale bar equals 25 μm.

Table 5. A summary of the present results

Frequencies	Early operations		Late operations
	GV-plus animal merogons	GV-minus vegetal merogons	GV-minus vegetal merogons
polyspermy:	low	high	low
cleavage:	high	zero	high
larval development:	low	zero	low
Epi-2 expression:	high	high	high



Frequencies	GV-plus merogons	GV-minus merogons	GV-minus merogons
polyspermy:	low	high	low
cleavage:	high	zero	high
larval development:	low	zero	low
Epi-2 expression:	high	high	high

Fig. 6. A summary of the present results. The development of animal and vegetal oocyte fragments produced prior to GVBD or during the early phase of GVBD suggests that factors required for normal fertilization and cell division may be localized in the peripheral GV nucleoplasm. Furthermore, these experiments suggest that epidermal determinants are present in the cytoplasm of a mature ascidian oocyte.

It has recently been shown that ascidian GVs contain a large pool of maternal RNA molecules that encode a proliferating cell nuclear antigen (PCNA). When Swalla and Jeffery (1996) examined the distributions of PCNA RNA and PCNA protein in the ascidian, *Styela clava*, PCNA RNA was detected in GVs and in the cytoplasm of postvitellogenic oocytes. PCNA protein was present throughout the ooplasm, excluding the cortical myoplasm. They observed that maternal PCNA RNA gradually decreased during cleavage and that zygotic PCNA transcripts were first detected by late gastrulation. The present results suggest there may be a rapid diffusion of PCNA transcripts associated with GV nucleoplasm that occurs during GVBD. The present results together with previous experiments demonstrating the androgenetic development of ascidian larvae (Minganti, 1959; Bates and Jeffery, 1987a) raise the possibility that GV nucleoplasmic factors may co-localize with other maternal determinants in the mature oocyte cytoplasm, and that the primarily function of the oocyte genome is to make these determinants. The present study examined the distribution of components required for the differentiation of an epidermal cell marker, Epi-2. In particular, the important question was addressed whether or not epidermal factors reside exclusively in the nucleoplasm of the ascidian oocyte. As previously discussed, the results of Conklin's cell lineage studies (1905) are consistent with the idea that a GV component may function in the autonomous determination of epidermal cell fates. The present results suggest that epidermal determinants are distributed in both the animal and vegetal hemispheres of a mature ascidian oocyte, as they are in the unfertilized eggs of *Ascidiella aspersa* (Dalcq, 1932), *Ascidia malaca* (Reverberi and Ortolani, 1962; Ortolani, 1958) and *Ciona savignyi* (Bates and Jeffery, 1988). When unfertilized eggs obtained from these species are cut into animal and vegetal egg fragments, both fragments can develop into larvae surrounded by epidermal cells. Furthermore, this conclusion is supported by cell fusion experiments using *Halocynthia roretzi* eggs (Nishida, 1994; Yamada and Nishida, 1996).

The expression of Epi-2 antigen in single-celled, polyspermic merogons produced from the vegetal hemisphere of oocytes that contained intact GVs, supports a previous experiment in which cleavage-blocked, single-celled zygotes expressed the Epi-2 antigen, indicating that normal cell division is not required for the differentiation of this epidermal marker (Nishikata *et al*., 1987). In conclusion, the results of the present study, together with previous self/nonself sterility results reported by Fuke and Numakunai (1996), suggest that nucleoplasmic factors associated with the ascidian GV are required for normal fertilization. The evidence suggesting that the nucleoplasm contains cell division factors is not as compelling as it is for fertilization factors if, as previously discussed, polyspermy directly inhibits normal cell division.Therefore, the search for cell division factors sequestered and subsequently released from urochordate GV nucleoplasm must await future experiments with protein and RNA probes to map, *in situ*, the movements of various kinds of cloned cell division factors during GVBD to compare with similar events in arthropods and vertebrates. The present results suggest that factors required for the differentiation of epidermal cells reside in the cytoplasm of mature ascidian oocytes, a key experimental result for future studies that will examine the molecular nature of urochordate epidermal determinants.

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